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Aerobic biodegradation of polyethylene glycols of different molecular weights in wastewater and seawater

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ABSTRACT

In order to distinguish between aerobic biodegradation of synthetic polymers in fresh and seawater, polyethylene glycols (PEGs) were systematically and comparatively investigated in inocula from municipal wastewater and seawater aquarium filters for the first time. The molecular weight (MW) of the PEGs, ($HO(CH_2CH_2O)_nH$, $n = 3$–1350) as representatives of water-soluble polymers, ranged from 250 to 57,800 Da. The biodegradation was observed by removal of dissolved organic carbon and carbon dioxide production by applying standardized ISO and OECD test methods. Specific analyses using liquid chromatography mass spectrometry (LC–MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were performed. All PEGs selected were completely biodegradable in freshwater media within 65 d. PEGs with an MW up to 14,600 Da have a similar degradation pathway which is characterized by gradual splitting of C_2-units off the chain resulting in formation of short-chain PEGs. In artificial seawater media, full biodegradation of PEGs up to 7400 Da required more time than in freshwater. PEGs with MW 10,300 and 14,600 Da were only partially degraded whereas PEGs with MW 26,600 and 57,800 Da were not degraded for a period of 135 d. The biodegradation pathway of PEG 250 and PEG 970 in seawater is similar to that for freshwater. For PEGs having an MW from 2000 to 10,300 Da, the degradation pathway in seawater differs from the pathway of the shorter PEGs.

1. Introduction

Little is known about microbial degradation of both water-insoluble and soluble synthetic polymers in marine environments due to the longevity of most synthetic polymeric structures. These materials are present in high proportion among marine debris worldwide (Derraik, 2002). Microscopic fragments of water-insoluble plastics are found in sediments of beaches and in estuarine and subtidal sediments, indicating a breakdown through mechanical action (Thompson et al., 2004). This has, however, to be distinguished from the biodegradation on molecular level. The water-insoluble poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), a particularly promising polyhydroxyalkanoate copolymer and subject of commercial interest, was found to be degraded modestly by a tropical marine bacterium cultivated in artificial seawater (Leathers et al., 2000). For other water-insoluble polymers, such as photodegradable polyethylenes (PEs) and poly(vinyl chlorides) (PVCs), degradation may start after abiotic processes such as photo-oxidation have occurred (Gardette et al., 1989; Andrady et al., 1993; Arnaud et al., 1994). This leads to short-chain polymeric structures and a shift in the molecular weight (MW) distribution.

Polyethylene glycols (PEGs, $HO(CH_2CH_2O)_nH$) are water-soluble non-ionic synthetic polyethers of ethylene oxide and are used in a wide range of applications. This includes the

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production of cosmetics, plastics, water-soluble lubricants, pharmaceuticals, antifreeze agents and non-ionic surfactants. The annual production of PEGs is millions of tons worldwide (Huang et al., 2005). After industrial utilization, a high proportion of them enter conventional wastewater treatment plants (WWTPs) (Huang et al., 2005). In general, water-soluble plastics are discarded via the sewage system after use and can reach the aquatic environment after wastewater treatment (Swift, 1993). Low molecular PEGs occur as metabolites of the biodegradation of polyethoxylated surfactants such as alcohol ethoxylates (Steber and Wierich, 1985; Marcomini et al., 2000) and PEG 550 (number indicates average MW) were found in river water and seawater between 0.5 and 68 $\mu g L^{-1}$ (Jonkers and de Voogt, 2003).

PEGs with MWs ranging from 200 to 40,000 Da have been extensively investigated for both aerobic and anaerobic biodegradation but mainly for freshwater media with microorganisms obtained from sources such as soil, river sediments and sludge of WWTPs. Aerobic degradation of PEGs in freshwater media was studied using microorganisms obtained from various sources. PEGs up to 20,000 Da MW were degraded by a strain occurring in soil (Haines and Alexander, 1975), PEGs up to 14,000 Da MW, but not PEG 20,000, were decomposed by bacteria isolated from river sediments (Obradors and Aguilar, 1991). Sewage bacteria were able to degrade PEG 4000 (Cox and Conway, 1976) whereas a facultative, mixed culture, obtained from a domestic WWTP and adapted to PEG 10,000, was able to metabolize this polymer but was incapable of utilizing either PEG 20,000 or PEG 35,000 (Otal et al., 1997). It was found that biodegradability generally decreased with increasing MW. A synergistic, mixed culture of *Flavobacterium* and *Pseudomonas* species was able to utilize PEGs of up to 20,000 Da MW and tetraethylene glycol (TEG, MW 194) was aerobic degraded leading to TEG-mono- and dicarboxylic acids, tri-, di and monoethylene glycol as metabolites, analyzed by gas chromatography mass spectrometry (GC–MS) (Kawai et al., 1978). Microbes isolated from the sludge of a WWTP efficiently decomposed PEG up to 20,000 Da in size, either under aerobic or anaerobic conditions (Huang et al., 2005). The efficiency of the aerobic process was much higher compared with that of the anaerobic process. Aerobic degradation of PEG 3400 by marine bacteria isolated from sediments of inter-tidal mudflats was reported for the fist time by (Pan and Gu, 2007). Unfortunately, the seven PEG 3400 degrading bacteria were cultivated in freshwater mineral salt media on agar plates and not in seawater media.

Anaerobic degradation of PEGs of up to 20,000 Da MW using freshwater media has been reported with bacteria isolated from anaerobic reactors of urban WWTPs (Dwyer and Tiedje, 1983, 1986; Otal and Lebrato, 2003). The anaerobic degradation of PEGs in seawater media was first mentioned using bacteria isolated from anaerobic mud of the Canale Grande, Venice, Italy (Schink and Stieb, 1983). Further studies showed that the anaerobic cultures in seawater media were able to metabolize PEGs with MWs in a range from 200 to 40,000 Da, most of them yielding acetate, acetaldehyde and ethanol (Straß and Schink, 1986).

Little is known about aerobic biodegradation of PEGs of all MWs, in seawater media. It is also not known if PEGs with MW higher than 20,000 Da are degraded under aerobic conditions in freshwater media by microorganisms found in sludge of conventional WWTPs. In nearly all the reports cited above, the amount of the parent compound PEG was quantified by absorptiometric and spectrophotometric analyses, thin layer chromatography or measurement of the total organic carbon (TOC). Metabolites, such as alcohols, volatile fatty acids, and methane, were assayed by gas chromatography with flame ionization detector (GC-FID). PEGs with chain lengths from 3 to 20 ethoxylate groups (MWs ranging from 150 to 900 Da), released during biodegradation of a linear alcohol ethoxylate blend in freshwater with inoculum from WWTP effluent, were measured with high performance liquid chromatography (HPLC) using fluorescent detection after derivatization (Marcomini et al., 2000). The fate of individual homologues was investigated; as a result, PEGs are biodegraded by hydrolysis and by oxidative hydrolysis, leading to shorter PEG oligomers and carboxylated PEGs, respectively (Marcomini et al., 2000). A study about aerobic biodegradation according to the River Water Die-Away Method using PEG 300 as parent compound found the same result (Zgoła-Grześkowiak et al., 2006). The behavior of PEG homologues with MWs higher than that of PEG 900 is unknown.

The aim of this work was to investigate the aerobic biodegradation of PEGs in a range from MW 250 up to 58,000 Da (repeating units of $n = 3$–1350) under simulated aerobic conditions in freshwater and artificial seawater media, using microorganisms contained in sludge of a conventional WWTP and marine microorganisms obtained from filters of a seawater aquarium, respectively. Standardized ISO and OECD guidelines for the testing of chemicals in aqueous media were applied. The characterization of the microorganisms was not performed. The decrease of PEG was observed by removal of the dissolved organic carbon (DOC), and carbon dioxide (CO_2) production. The fate of individual homologues of PEG was investigated by specific analyses using liquid chromatography mass spectrometry (LC–MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Based on the obtained data, a comparison of the pathway of PEG degradation in freshwater and seawater media was possible.

2. Materials and methods

2.1. *Biodegradation tests*

The biodegradation tests were performed using two different test systems. One was the CO_2 Evolution Test (modified Sturm Test) according to the guidelines OECD 301 B and ISO 9439 which determines the ultimate biodegradability of organic compounds by aerobic microorganisms in water, using a static aqueous test system as described by Strotmann et al. (2004). The other static test system used was the combined CO_2/DOC Test in compliance to OECD 301 A and B and ISO 9439 Annex D (Strotmann et al., 1995). In both test systems, the biologically produced CO_2 was used as the analytical parameter to calculate the degree of biodegradation. Additionally for the combined CO_2/DOC Test, the DOC of the solution is measured to calculate the removal of DOC. The combination

of both measurements allows a prediction of biodegradation and abiotic removal of a test substance, e.g. by adsorption to activated sludge.

The biodegradation tests were carried out in freshwater, using municipal activated sludge from the WWTP of the city Mannheim (Germany), and in artificial seawater (ISO 16221, OECD 306), using marine microorganisms acquired from filters taken out of a seawater aquarium at Luisenpark, Mannheim (Germany). An average daily flow of about 100,000 m^3 WW, 45% municipal and 55% industrial, is treated in the WWTP Mannheim. The activated sludge was collected from the aeration tank of the plant and preincubated up to 2 d at a temperature of 22 ± 2 °C, while the sludge suspension was constantly aerated to reduce the extent of background CO_2 production before being applied to the test system. One day before use, a suitable aliquot of the activated sludge suspension was washed with tap water, sieved by a finely woven mesh (mesh size nominally 1 mm) and pre-aerated for about 24 h. The sludge suspension was adjusted to a concentration of 6.0 g L^{-1} of dry weight and then added to the test vessels to obtain a sludge concentration of 30 mg L^{-1} dry substance. The marine inoculum was obtained from the filter unit of a seawater aquarium. The filters were gently squeezed and the inoculum suspension was then diluted with artificial seawater. The suspension was filtered using a sieve (mesh width approximately 0.70 μm) and aerated 22 ± 2 °C in a dark room for up to 7 d until use. Mixtures of the test substance, a defined inorganic mineral medium (OECD 301A and B, OECD 306, ISO 9439 and ISO 16221), and the non pre-adapted inoculum were incubated in freshwater and seawater, respectively, and aerated with CO_2-free air at a temperature of 22 ± 2 °C for at least 28 d (depending on the progress of the degradation). The initial concentration of each PEG as the sole source of carbon was 20 mg L^{-1} referring to the DOC. Each test series was done twice in two parallel vessels. In addition to the test substances, blank values, a reference substance (aniline for freshwater and sodium benzoate for seawater) and inhibition of the inoculum were studied. Samples of all test systems were collected on the day the test started (day 0) and then 2–3 times a week. Measurements for evolved CO_2 and DOC were performed on day of sampling. Samples for MS analyses were frozen at −20 °C until analysis.

2.2. *Reagents and chemicals*

Nine PEGs covering an MW range from 250 to 58,000 Da with polydispersity and molecular characteristics as shown in Table 1 were obtained from BASF (Ludwigshafen, Germany). Aniline, sodium benzoate, mineral salts for the test media, sodium hydroxide and potassium hydroxide were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (Buchs, Switzerland). PEGs standards from Polymer Standards Service (Mainz, Germany) were used for gel permeation chromatography (GPC). Acetic acid (100%, p.a.), ammonium acetate (p.a.), acetonitrile and methanol (both Suprasolv grade) were purchased from Merck (Darmstadt, Germany). Amberlite UP6040 mixed-bed resin, sodium trifluoroacetate (NaTFA), lithium trifluoroacetate, potassium trifluoroacetate, silver trifluoroacetate and trifluoroacetic acid (TFA) were from Carl Roth (Karlsruhe, Germany). *trans*-2-[3-(4-*tert*-Butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB), α-4-hydroxycinnamic acid (HCCA), di(ethylene glycol) ethyl ether and triethylamine were obtained from Sigma–Aldrich (Buchs, Switzerland). Ultrapure water was prepared by a Milli-Q system (Millipore, Milford, MA, USA).

Table 1 – Molecular weight distributions of the PEGs used, obtained by gel permeation chromatography (n = 2)

Name of PEG	Molecular characteristics				
	M_n [Da]	M_w [Da]	M_p [Da]	D	n
PEG 250	120	251	275	2.10	5
PEG 970	799	974	995	1.22	22
PEG 2000	1424	2009	2114	1.41	45
PEG 4500	3967	4519	4401	1.14	102
PEG 7400	5444	7426	7550	1.36	168
PEG 10,300	8051	10,310	10,599	1.28	234
PEG 14,600	10,909	14,629	14,846	1.34	332
PEG 26,600	15,548	26,642	25,807	1.72	605
PEG 57,800	25,075	57,759	45,161	2.30	1,312

M_w, weight-average molecular weight; M_n, number-average molecular weight; M_p, peak maximum molecular weight; D, polydispersity; and n, average degree of ethoxy-units.

2.3. *Sample preparation*

In general, seawater samples were desalted by the following procedure: to 400 mg Amberlite UP6040 mixed-bed resin, 500 μL sample were added and shaken on a Vortex shaker for 1 min. The ratio of ion exchanger to sample was used for any sample volume. For MALDI-TOF-MS, the prepared seawater and untreated freshwater samples could be used without further preparation. For GPC and LC–MS analyses, all samples were filtered using Spartan® 0.45 μ syringe filters before analysis. LC–MS samples were measured either diluted 1:5 with Milli-Q water (freshwater samples) or without dilution (desalted seawater samples). For characterization by GPC, solutions of each PEG used were prepared with 3 g L^{-1} in 0.2% TFA in Milli-Q water and measured.

Preparations for MALDI-TOF-MS were carried out on a conventional MALDI target with 26 spots from Bruker Daltonik (Bremen, Germany). Two matrices with two preparation techniques were applied. DCTB was used in thin layer preparation for PEGs with MW < 8000 Da due to its very low matrix interferences. HCCA was applied in dried droplet preparation for PEGs with MW > 8000 Da because DCTB showed low intensities and poor resolution for these PEGs. Other matrices, such as sinapinic acid, 2,5-dihydroxybenzoic acid, *trans*-retinoic acid, dithranol and 3-indoleacrylic acid, were also tested but the mass spectra obtained showed less resolution and/or lower sensitivity for higher MW compared to mass spectra with DCTB and HCCA. The concentration of DCTB was 20 g L^{-1} in methanol, HCCA was used as saturated solution in 0.1% TFA in Milli-Q water:acetonitrile = 2:1 (v:v). NaTFA was applied as cationization agent with 10 g L^{-1} in methanol:Milli-Q water = 1:1 (v:v) because PEG–Na adducts gave the strongest intensities in comparison with potassium trifluoroacetate and lithium trifluoroacetate, which were also tested.

Thin layer preparation was performed as follows. DCTB solution was applied to the MALDI target (0.5 μL) and air-dried to give a thin layer. 10 μL of the aqueous PEG sample and 0.5 μL of the NaTFA solution were added together, mixed and 1.0 μL was pipetted on the DCTB thin layer. For dried droplet preparations, 10 μL of the HCCA solution, 10 μL of the PEG sample and 0.5 μL of the NaTFA solution were added together, mixed and 1.0 μL was applied to the MALDI target. The measurements were carried out immediately after evaporation of the solvents.

2.4. *Measurement of evolved CO_2 and DOC and calculation the degree of biodegradation*

The determination of biologically formed CO_2 and DOC was done as described by Strotmann et al. (1995, 2004). The measured amount of CO_2 was compared with the calculated maximal theoretical production of CO_2 and indicated as degree of biodegradation in percent. The removal of DOC was calculated based on the amount of DOC at the beginning of the test and expressed as a percentage. DOC and dissolved inorganic carbon (DIC) analyses were performed with a Shimadzu 5000A and Shimadzu V-CSN TOC analyzer with a limit of detection (LOD) of 50 $\mu g\,L^{-1}$.

2.5. *GPC, LC–MS and MALDI-TOF-MS analyses*

A molecular characterization of the PEGs used was performed on a conventional GPC system, consisting of a Compact Pump 2250 (Bischoff, Leonberg, Germany), degasser ERC-3322 (Erma, Tokyo, Japan), injection valve Rheodyne 7125 (Rohnert Park, CA, USA) and a refractive index detector Shodex RI-71 (Showa Denko K.K., Tokyo, Japan). The separation was performed on a PSS Suprema Linear M column, 8 × 300 mm, 10 μ, 1000 Å (Polymer Standards Service, Mainz, Germany). 100 μL of the sample solution were injected into the GPC system, 0.2% TFA in Milli-Q water at 1.0 mL min^{-1} was used as the mobile phase. The column temperature was set at 22 °C whereas the internal temperature of the detector was set at 35 °C. A series of PEG standards with narrow MWs ranging from 232 to 1,010,000 Da was used to calibrate the GPC system. Diethylene glycol ethyl ether was used as internal standard to compensate shifts in retention time.

LC–MS analyses were performed on a PE Sciex API 150 MCA (Norwalk, CT, USA) with a Hypersil MOS-1 column, 250 × 2.1 mm, 5 μm (Thermo, Bellafonte, PA, USA). The injection volume was 50 μL and the flow rate was 200 μL min^{-1}. Eluent A consisted of water/acetonitrile (95:5; v:v) and eluent B was water/acetonitrile (20:80; v:v), both eluents contained 5 mM acetic acid and 5 mM triethylamine. Separation for PEG 250 was achieved by isocratic mixture of 90% eluent A and 10% eluent B whereas for PEG 970 a gradient decreased from 80% A to 70% A in 12 min, then was re-established to initial conditions within 5 min and equilibrated for 20 min. A drying gas flow (nitrogen, 7 L min^{-1}) was applied and measurements were carried out in the Single Ion Monitoring (SIM) mode to detect ions using positive electrospray ionization (ESI). Individual PEG chains were detected as positive PEG–Na adducts.

MALDI-TOF mass spectra were obtained using a Bruker Daltonik (Bremen, Germany) Biflex III MALDI-TOF mass spectrometer equipped with a scout 26, an 1 GHz/channel digitizer and a N_2-laser ($\lambda = 337$ nm) operating at a pulse rate of 6 Hz. Positive ions were accelerated with pulsed ion extraction by a voltage of 19 kV in reflector mode and 20 kV in linear mode, respectively. PEGs with MW < 8000 Da were analyzed in the reflector mode and PEGs with MW > 8000 Da in the linear mode. The ions were detected using a micro-channel plate detector. Calibration of the instrument was carried out before each measurement with the same PEG as was used in the degradation test.

3. Results and discussion

3.1. *Molecular weight distribution of PEGs used*

In order to obtain the molecular weight distribution of the PEGs used in this study, all PEGs were analyzed by GPC. Table 1 shows the molecular characteristics specified as weight-average MW (M_w), number-average MW (M_n), peak maximum MW (M_p), and polydispersity D, calculated as the ratio of M_w/M_n. The measured values for M_w were used to specify the PEGs in this study. The polydispersity was in the range 1.14–2.30 indicating a narrow MW distribution obtained by a unimodal distribution.

3.2. *Biodegradation tests*

The aerobic biodegradation of PEGs having MWs from 250 to 57,800 Da under simulated conditions in both freshwater and seawater media was tested. Fig. 1a shows the results ($n = 2$) of the combined CO_2/DOC Test obtained from degradation studies in freshwater with WWTP sludge inoculum. All PEGs with MW up to 57,800 Da are biodegradable under the selected conditions. The PEGs can be divided into two groups: one group, the PEGs with MW from 250 to 14,600 Da, was fully biodegraded within 20 d. They had a lag-phase of up to 5 d; their graphs in the combined CO_2/DOC Test (Fig. 1a) are similar in evolution with a DOC removal of 14% per day from day 2–7. No differences concerning the biodegradation could be seen for these PEGs. The second group includes the PEGs 26,600 and 57,800, which had a longer lag-phase of approximately 22 d and were completely degraded within 45 d and 65 d, respectively. Evidently, the biodegradation of PEGs having an MW > 14,600 Da required much more time than that of PEGs with shorter chains but even PEG 57,800 was entirely biodegradable. For PEGs up to MW 57,800 Da in freshwater media and the microorganisms used, the velocity of PEG biodegradation generally decreases with increasing MW while the ultimate % level of biodegradation remains at >90% DOC removal. This is a new result because it is generally accepted that the level of PEG biodegradation in freshwater decreases with increasing MW (Haines and Alexander, 1975; Watson and Jones, 1977; Dwyer and Tiedje, 1983; Otal et al., 1997).

Aerobic biodegradation of PEGs in artificial seawater using marine microorganisms is possible (Fig. 1b) but there are differences compared to freshwater media. The graphs of PEG 250, 970 and 2000 have the same trend with a lag-phase of not more than 6 d and a DOC removal of 10% per day from day 7–

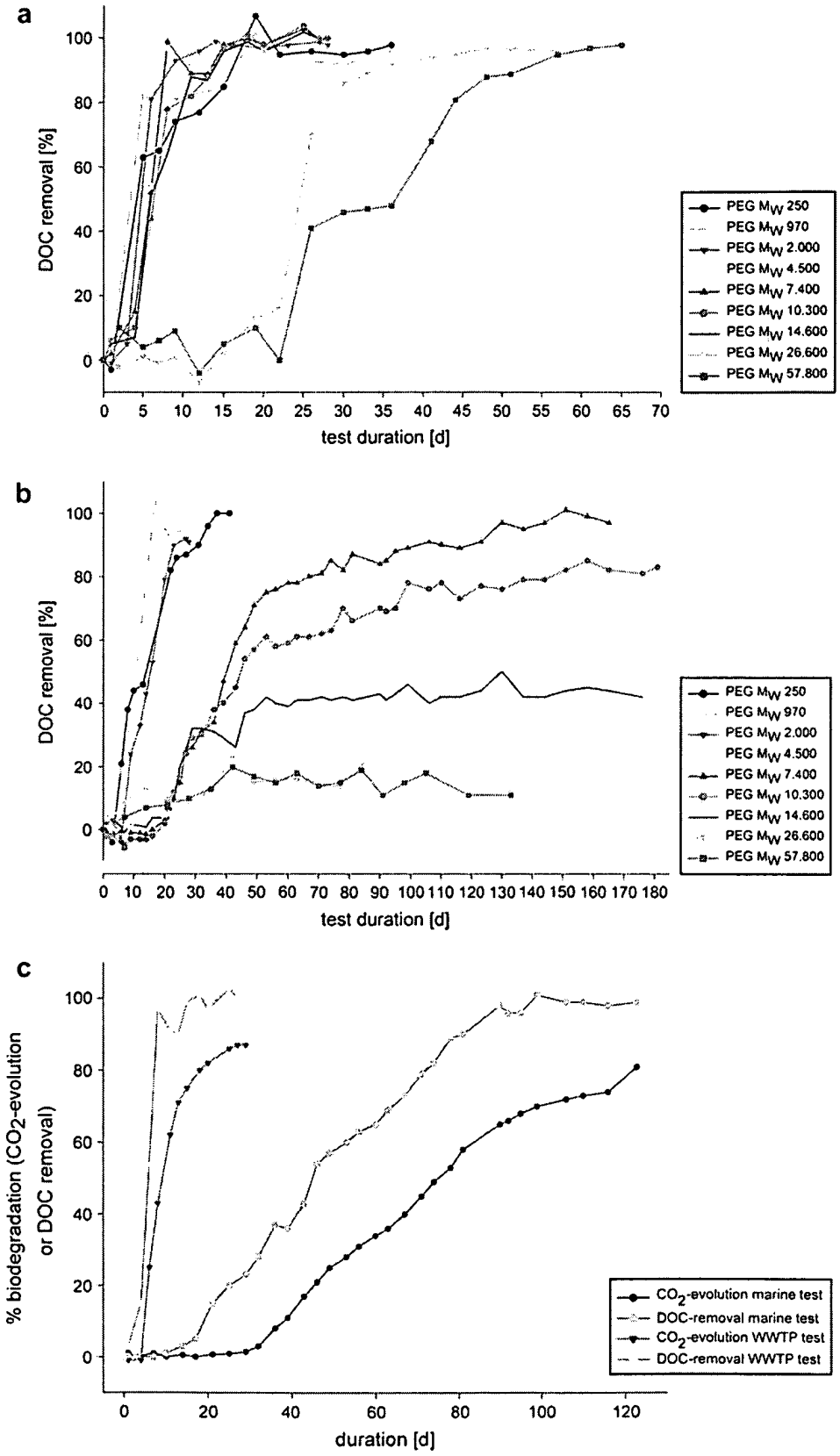

Fig. 1 – Aerobic biodegradation (expressed as DOC removal in %) of PEGs. (a) In freshwater using WWTP sludge inoculum, 0–65 d ($n = 2$). (b) In artificial seawater using marine inoculum, 0–180 d ($n = 2$). (c) Comparison of DOC removal and CO_2 evolution in both media for PEG 4500, 0–120 d ($n = 2$). Results were obtained from the combined CO_2/DOC Test.

15, which is similar to freshwater media. These short-chain PEGs were fully biodegraded within 37 d. The PEGs with longer chains, PEG 4500, 7400, 10,300 and 14,600, all had a lag-phase of around 20 d until the biodegradation started. The longer lag-phase may result from inoculum of a seawater aquarium with non-adapted microorganisms whereas the microorganisms from WWTP sludge inoculum were probably adapted to PEG. The graphs of PEG 4500–14,600 are quite similar initially but then they vary, leading to different results for their biodegradation potential. PEG 4500 is completely degraded after approximately 100 d whereas PEG 7400 needs around 130 d. PEG 10,300 has reached only 80% DOC removal after 180 d and the degradation of PEG 14,600 has terminated after 50 d when DOC removal exceeded 40%. Thus PEG 10,300 and 14,600 are not completely degraded in seawater media after 180 d, which is in contrast to the freshwater media in which they were entirely biodegraded within 20 d. The biodegradation stops at distinct % levels of DOC removal and the oligomeric distribution of the PEGs may imply, that only short-chain PEGs as part of PEG 10,300 and 14,600 were biodegraded. The higher the MW of a PEG, the lower the amount of short-chain PEGs. Indeed, PEG 26,600 and 57,800 were not degraded in seawater for a period of 135 d, indicating the absence of biodegradable low molecular PEGs. Neither significant DOC removal nor CO_2 production was observed (Fig. 1b). This is contrary to freshwater media in which an extensive biodegradation of these PEGs could be seen. The reference compound sodium benzoate in the reference test systems of PEG 26,600 and 57,800 was completely degraded within 16 d indicating an active microbial population at the beginning of the test series. As a result of PEG degradation in seawater media, the time required to reach a certain % level of biodegradation increases, whereas the biodegradability decreases with increasing MW. This was found to be the case in several studies conducted in freshwater media (Haines and Alexander, 1975; Watson and Jones, 1977; Dwyer and Tiedje, 1983; Otal et al., 1997) and can be transferred to seawater media.

The results based on evolved CO_2 from CO_2 Evolution Test and combined CO_2/DOC Test were similar for both test systems. The evolved CO_2 production did not reach 100% of calculated theoretical CO_2 production, but was in a range between 70 and 95%. Fig. 1c shows results for PEG 4500 in both media expressed as DOC removal and CO_2 evolution. The lag-times of graphs based on CO_2 evolution were always longer than those of graphs created by DOC removal with differences being in the region of 1–3 d. This finding should be considered in tests of water-insoluble polymers when no DOC can be measured.

The biodegradation tests are based on the determination of sum parameters. With the results obtained, it is possible to draw conclusions about the differences in biodegradation of PEGs in freshwater and seawater media in terms of time and chain length. It is impossible, however, to compare the degradation pathways of PEG with similar MW-distributions in both environmental compartments. For this purpose, the fate of individual homologues of PEG was investigated by specific mass spectrometric analysis enabling a comparison of the pathway of PEG degradation in freshwater and seawater media.

3.3. *Detection of individual PEG homologues by mass spectrometry*

The specific analysis of single PEG homologues having a repeating unit of 44 Da was performed with both LC–MS and MALDI-TOF-MS. Freshwater samples could be measured without any sample preparation whereas seawater samples had to be desalted. No enrichment was necessary; the concentration of, for example, PEG 970 at approximately 37 mg L^{-1} was sufficient for mass spectrometric analysis. Due

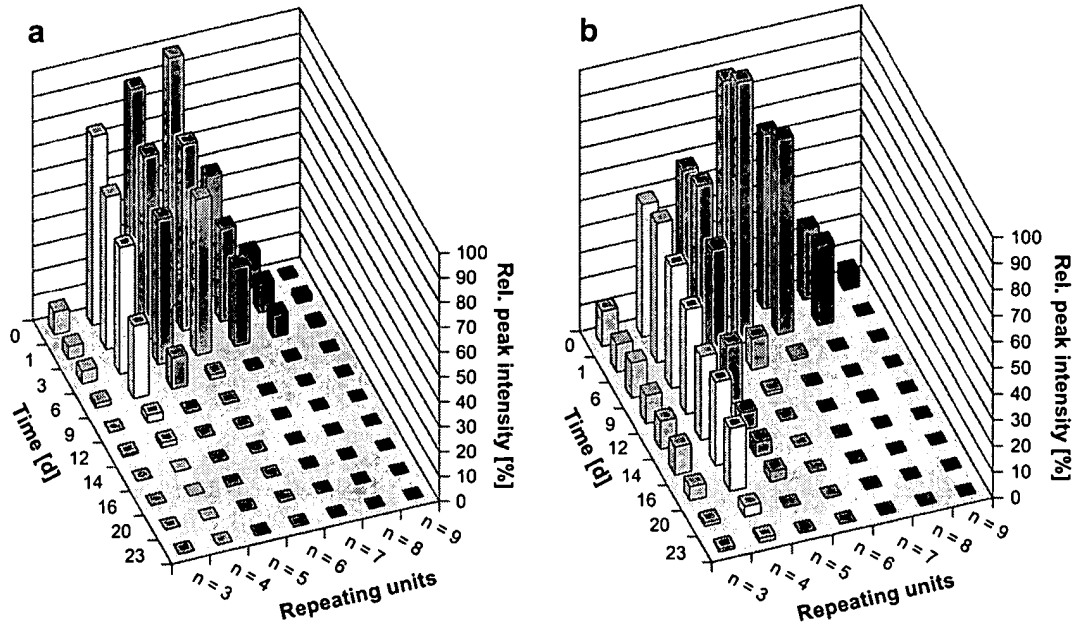

Fig. 2 – Fate of individual homologues during aerobic biodegradation (0–23 d) of polydispersed PEG 250, measured by (+)-ESI-LC–MS. (a) Freshwater media, (b) artificial seawater media. Peak intensities are set in relation to the highest peak intensity detected during all measurements.

to the limited mass range of the quadrupole mass analyzer, only PEG 250 and 970 could be measured with this LC–MS system after chromatographic separation. For higher MW PEGs, MALDI-TOF-MS was applied as described above.

3.4. *Results obtained for PEG homologues analyzed by LC–MS*

The fate of each homologue of polydisperse PEG 250 and PEG 970 for freshwater and seawater media is shown in Figs. 2 and 3. The peak intensity of each PEG homologue, measured as PEG–Na adduct by (+)-ESI-LC–MS, was set in relation to the peak intensity of the PEG homologue with the highest intensity. This illustration is limited due the different detector responses for the homologues of PEGs. Depending on the incubation time, the change in intensities of each PEG homologue can be observed. At the beginning of the DOC removal test, the intensities of all homologues show the typical pattern of a polydispersed mixture. As can be seen for both media, the degradation of PEG 250 (Fig. 2) and PEG 970 (Fig. 3) started simultaneously with all PEG chains leading to a shift in the MW distribution towards the short-chain homologues. The long-chain PEGs are completely degraded by gradual splitting of C_2-units off the chain. This is a common accepted pathway of aerobic PEG degradation in freshwater media (Kawai et al., 1978; Balson and Felix, 1995; Gu, 2003), which now can be transferred to seawater media for PEG 250 and PEG 970. During degradation, short-chain PEG homologues are generated from the long-chain PEGs (Figs. 2 and 3). The amount of short-chain PEG increases in both media and decreases again when

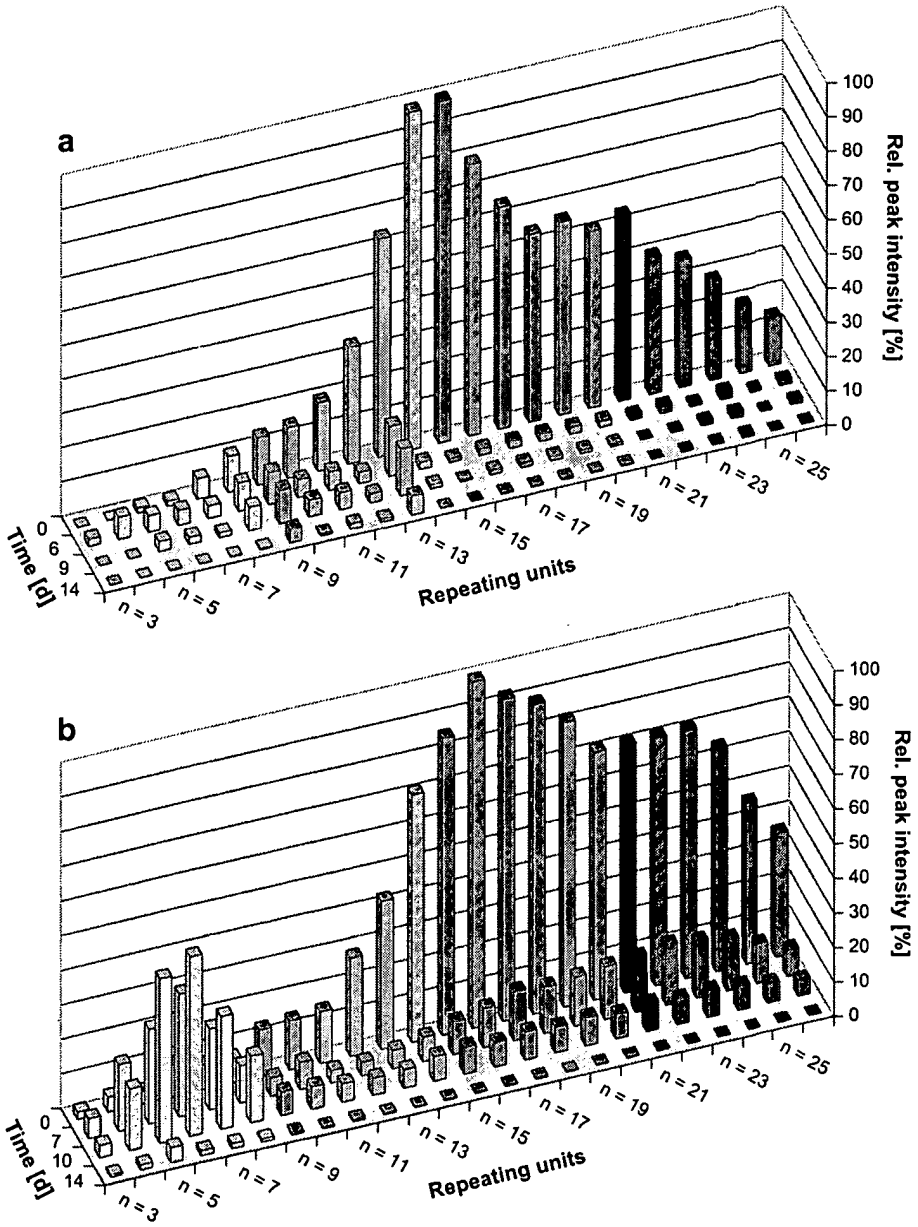

Fig. 3 – Fate of individual homologues during aerobic biodegradation (0–14 d) of polydispersed PEG 970, measured by (+)-ESI-LC–MS. (a) Freshwater media, (b) artificial seawater media. Peak intensities are set in relation to the highest peak intensity detected during all measurements.

degradation proceeds (Fig. 3). The aerobic biodegradation of polydispersed PEG 300 in freshwater with formation of short homologues is known (Zgoła-Grześkowiak et al., 2006). However, we found out not only the biodegradation of PEG 250 occurs in the same way for freshwater and seawater media but also PEG 970. Although the microorganisms are assumed to be different since we have not defined the microbial population, the biodegradation of PEG 250 and PEG 970 in seawater and freshwater seem to be similar with the only difference the biodegradation in freshwater media being faster. This result coincides with that reported for biodegradation of alkylphenol ethoxylates and alkylbenzene sulfonates in seawater (Jonkers and de Voogt, 2003; León et al., 2004).

When searching for metabolites of PEG 250 and PEG 970, molecules having repeating units of 44 Da were found. The molecules had an *m/z* of either 2 less or of 14 more than the *m/z* of corresponding PEG–Na adducts. The intensities of these metabolites increased during biodegradation and then decreased again (data not shown). This suggests the oxidation of one terminal OH-group to the corresponding aldehyde has occurred, which is the initial step of the biodegradation of PEG 250 and PEG 970 (Kawai et al., 1978; Gu, 2003). Further oxidation leads to the carboxylic acid derivative (Kawai et al., 1978; Gu, 2003).

3.5. *Results obtained for PEG homologues analyzed by MALDI-TOF-MS*

Fig. 4 shows the results of the aerobic biodegradation of PEG 2000 in freshwater media measured by MALDI-TOF-MS. On day 1 (Fig. 4a), each individual PEG–Na-homologue (beside PEG–K adducts and doubly charged PEG–2Na adducts) of the

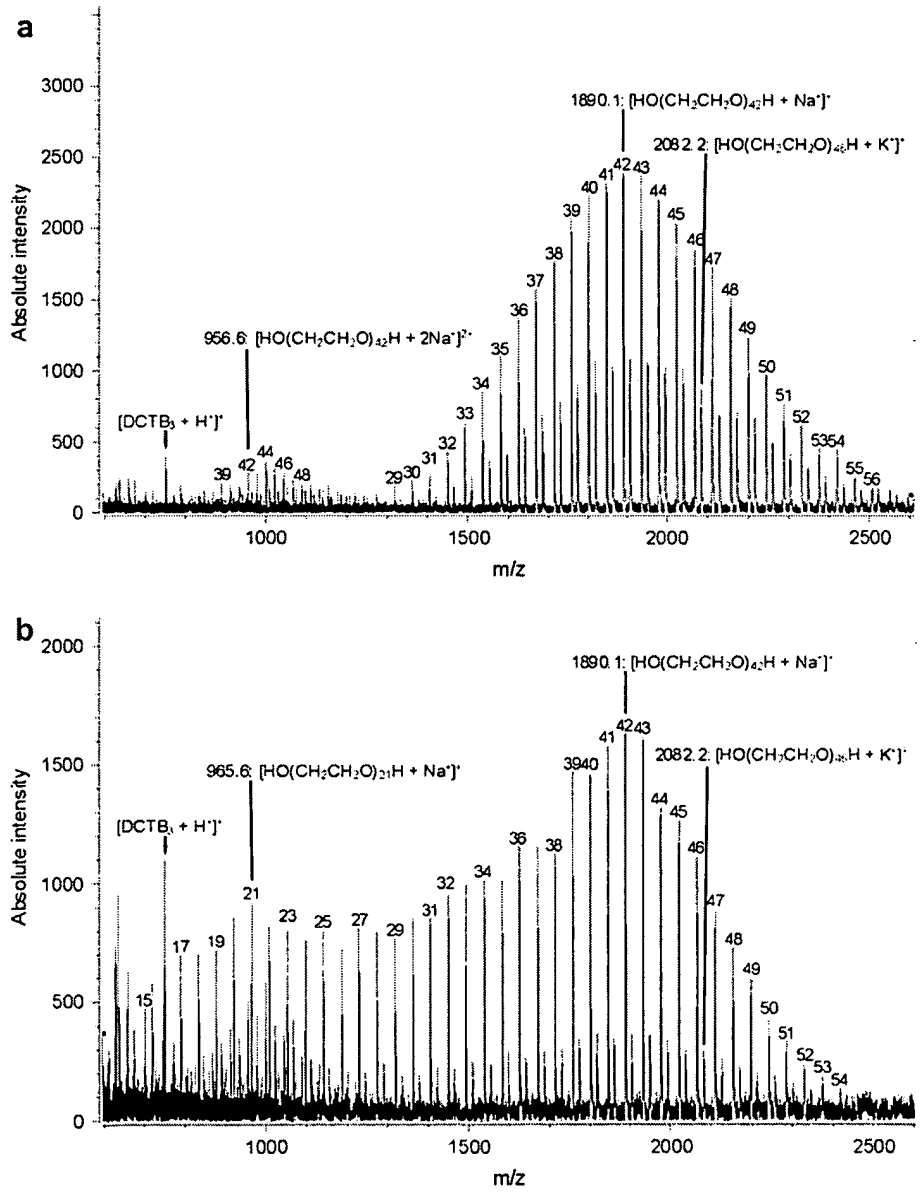

Fig. 4 – MALDI-TOF-MS spectra of polydispersed PEG 2000 during aerobic biodegradation in freshwater media with WWTP sludge inoculum, matrix was DCTB. (a) Sample from day 1, (b) sample from day 6. Numbers indicate repeating units.

polydispersed PEG 2000 can be seen in the spectrum, covering an *m/z* range from 1300 to 2600. When the biodegradation is in process on day 6 (Fig. 4b), a shift in the chain length and MW of the former PEG 2000 has occurred. Individual PEG homologues cover an *m/z* range from 600 to 2500, indicating a loss of long-chain PEGs and a formation of short-chain PEGs. On day 9 (data not shown), PEG could no longer be detected. Obviously, the biodegradation of PEG 2000 is similar to that of PEG 250 and 970 for the freshwater media with formation of short-chain homologues.

In artificial seawater, a similar spectrum (Fig. 5a) to that for freshwater media (Fig. 4a) on day 1 was obtained for PEG 2000, with the difference that only singly and doubly charged PEG–Na adducts were detected due the desalting of the sample and adding of NaTFA as cationization agent. Although the DOC removal has reached some 50% on day 14 (Fig. 1b), no short-chain PEGs were detected at this time (Fig. 5b). This is in contrast to the degradation of PEG 2000 in freshwater in which short-chain homologues were found (Fig. 4b). Comparing Fig. 5b with Fig. 5a, a loss of some individual PEG homologues in the *m/z* range 1300 to 1600 can be observed but the long-chain PEGs are still present. Other samples of PEG 2000 (day 12 and day 16, data not shown) were analyzed, showing the same distribution of long-chain homologues. On day 20, only PEGs having an MW between 1900 and 2700 Da were present (data not shown), short-chain PEGs could not be detected. Evidently, PEG homologues with MW < 1900 Da are degraded preferentially prior to long-chain homologues. The degradation of PEG 2000 is as fast as that of PEG 250 and PEG 970 in seawater media and was complete after 27 d (Fig. 1b). A complete degradation of PEG 2000 without detecting smaller polymer intermediates may imply that another complex mechanism is participated in the degradation process of such polymers. Taking into account the lower number and activity of marine

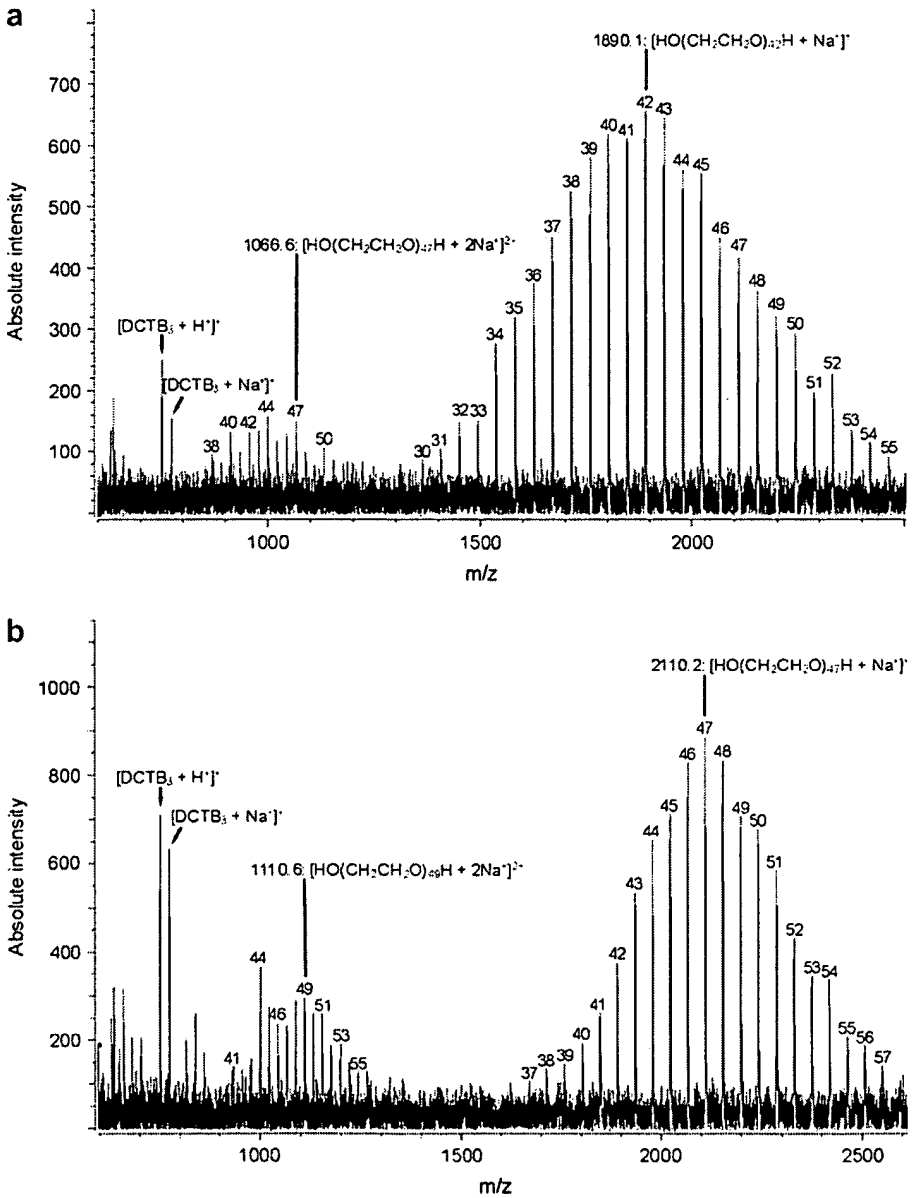

Fig. 5 – MALDI-TOF-MS spectra of polydispersed PEG 2000 during aerobic biodegradation in artificial seawater media with marine inoculum, matrix was DCTB. (a) Sample from day 1, (b) sample from day 14. Numbers indicate repeating units.

microorganisms especially towards longer chain homologues resulting in a slower turnover rate of such molecules, short-chain intermediates seem to be preferably degraded, immediately after their formation. Thus the detection of the missing intermediates could be limited due to a low concentration.

MALDI-TOF-MS spectra of PEG 4500, PEG 7400, PEG 10,300 and PEG 14,600 were recorded for freshwater media. All PEGs mentioned show a shift towards short-chain PEGs when the biodegradation is in progress (data not shown), meaning that PEGs from MW 250 to 14,600 all have a similar degradation pathway in freshwater, which is characterized by gradual loss of one oxyethylene group resulting in formation of short-chain PEGs. During degradation of PEGs 4500 to 10,300 in artificial seawater media, no short-chain PEGs were formed and detected using MALDI-TOF-MS (data not shown) what suggests a similar degradation pathway as that of PEG 2000 in seawater. The long-chain PEGs were present until the biodegradation has come to an end, no change in the distribution pattern was observed. On day 90, PEG 10,300 is disappeared (data not shown), indicating a complete primary degradation of this PEG in artificial seawater under the simulated conditions.

4. Conclusions

All of the selected PEGs in a range from MW 250 up to 58,000 Da are completely biodegradable regardless of the MW in freshwater media under aerobic conditions using microorganisms obtained from WWTP sludge. In seawater media with marine microorganisms, PEGs up to 7400 Da are entirely biodegradable whereas PEGs having higher MWs are only partially degradable and persistent to microbial attack, respectively. Our results show that under marine conditions the level of biodegradation decreases, while the lag time increases with increasing MW. Further studies for freshwater media may examine if PEGs with higher MW than 58,000 Da show a decrease in biodegradability when MW increases. Under both environmental conditions it appears that the time required for degradation generally increases with increasing MW.

In freshwater media the experiments indicate similar degradation pathways for PEG with MW 250 to 14,600 Da, which is characterized by formation of shorter homologues. A similar pathway is seen for PEGs having an MW < 1900 Da in seawater media, with the only difference the biodegradation in freshwater media being faster. Future studies may investigate if the microorganisms involved in the biodegradation in freshwater media are different from those in seawater media although the degradation pathway is similar. When the MW of the PEGs exceeds 1900 Da, the pathway in seawater media changes. PEGs having an MW from 2000 to 10,300 Da show probably a different biodegradation pathway with a stable MW pattern during degradation. The degradation of PEGs with higher MWs is limited in seawater media, no pathway can be given. Taking into account that no report has been found on the accumulation of PEGs in nature, it can be assumed that both high molecular PEGs have not yet been measured in marine habitats and their degradation in seawater may occur after a breakdown to shorter homologues influenced by biotic processes in discharge systems or abiotic processes.

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